ESTIMATION OF PLASMA ANTIOXIDANTS – VITAMIN E AND VITAMIN C IN ORAL PRE-CANCER AND CANCER PATIENTS

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Certificate

This is to certify that the Dissertation entitled **"ESTIMATION OF PLASMA ANTIOXIDANTS – VITAMIN E AND VITAMIN C IN ORAL PRE-CANCER AND CANCER PATIENTS",** done by **Dr. L. NATARAJAN**, Postgraduate student, M.D.S Branch-IV, Oral and Maxillofacial Pathology, Meenakshi Ammal Dental College and Hospital, Chennai, submitted to The TamiNadu Dr. M.G.R Medical University in partial fulfillment of the Master of Dental Surgery Degree examination in March 2007, is a bonafide research work done under my guidance and supervision.

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INTRODUCTION

Oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the former, leads to many biochemical changes and is an important contributing factor in several human chronic diseases including cancer. This oxidative damage results largely due to the formation and activity of free radical **(Balz Frei 1999)**[4]

Free radicals are molecules containing single unpaired electron. They are unstable and highly reactive. In an attempt to achieve stability, free radicals attack neighboring molecules to obtain another electron and in doing so, damage those molecules. The highly reactive unpaired electron is passed from one molecule to another neutralizing the recipient. This becomes a chain reaction that damages all macromolecules including proteins, carbohydrates, lipids and nucleic acids. The most important free radicals are oxygen derivatives, particularly superoxide and hydroxyl radical. Free radical formation occurs in the body by several mechanisms, involving both endogenous and environmental factors.

Oxidative damage, another name for the chemical reaction that free radicals can lead to a breakdown or even hardening of lipids, which makeup all cell walls. If the cell wall is hardened (lipid peroxidation), then it becomes impossible for the cell to properly get its nutrients, get signals from other cells to perform an action and many other cellular activities can be affected. In addition to the cell walls, other biological molecules are also susceptible to damage, including RNA, DNA and protein enzymes.

The primary site of free radical damage is the DNA found in the mitochondria. Mitochondria are small membrane-enclosed regions of a cell which produce the chemicals a cell uses for energy. Mitochondria are the "energy factory" of the cell. Every cell contains an enormous set of molecules called DNA which provide chemical instructions for a cell to function. This DNA is found in the nucleus of the cell, which serves as the "command center" of the cell, as well as in the mitochondria. The cell automatically fixes much of the damage done to nuclear DNA. However, the DNA in the mitochondria cannot be readily fixed. This free

radical generation process can disrupt all levels of cell function.

Methylation of cytosines in DNA is important for the regulation of expression of many genes **Helen wiseman and Barry Halliwell (1996)**[5] In free radical injury, the normal methylation pattern is altered, thus altering the genetic makeup causing mutation. Endogenously generated Reactive oxygen species (ROS), such as peroxides and oxygen free radicals play a major role in carcinogenesis.

The human body has several mechanisms to counteract the damage caused by free radicals; this is either by enzymes like glutathione peroxidase, superoxide dismutase, and catalase, or by antioxidants.

Antioxidants are substances when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate **(IS Young, JV Woodside 2001)**[84]**.**They can neutralize free radicals by accepting or donating an electron to eliminate the unpaired electron. Typically, this

means that the antioxidant molecule becomes free radical in the process of neutralizing a free radical to a non free radical molecule.

The non- enzymatic antioxidants include the lipid soluble vitamins (vitamin-E and vitamin-A) and water soluble vitamin-C. Vitamin-E has been described as the major chain breaking antioxidant in humans because of its lipid solubility. Vitamin-E is located in cell membranes, where it interrupts lipid peroxidation and may play a major role in modulating intracellular signaling pathway that relay on reactive oxygen species. Vitamin E can also directly quench ROS, including superoxide radical, OH, and O_2. Vitamin–E plays a primary defense against lipid peroxidation and oxidation.

Vitamin-C is the most abundant water soluble antioxidant in the body. It directly scavenges oxygen radical, hydroxyl radical and hydrogen peroxide. Vitamin-C neutralizes oxidants from stimulated neutrophils. Vitamin-C contributes to the regeneration of vitamin-E to its active

form. Vitamin-C is the most important compound implicated in recycling of alpha- tocopherol radicals.

The efficiency of antioxidants in prevention and treatment of oral cancer is controversial. Considerable laboratory evidence from chemical, cell culture, and animal studies indicate that antioxidants can possibly prevent the development of cancer. However recent clinical trials reached inconsistent conclusions.

Therefore, this study was conducted to evaluate plasma levels of non enzymatic antioxidants (vitamin-E and vitamin-C) of normal patients, patients with habits without any lesion, patient with habits having oral pre cancer, and having oral cancer by spectrophotometric analysis.

AIMS AND OBJECTIVES

1. To estimate the plasma levels of non- enzymatic antioxidants (Vitamin-E and Vitamin-C) in the following group of individuals:
    a. Individuals without tobacco-related habits or lesions
    b. Individuals with tobacco related habits but with out oral lesions
    c. Patients having tobacco related habits with oral pre cancerous lesions and conditions like Leukoplakia and oral sub mucous fibrosis.
    d. Patients having tobacco related habits with histologically confirmed Oral Squamous cell Carcinoma.

2. To compare the plasma antioxidant levels of Vitamin E and Vitamin C in the above groups.

REVIEW OF LITERATURE

FREE RADICALS:

Peter H. Proctor and Edward S. Reynolds (1984)[57] Free radicals are activated electronic species that are produced in biological system as antimicrobial defense.

UN Das (1990)[19] considers free radicals as molecules containing unpaired electron in the outermost orbital. The unpaired electron of the free radical accounts for the strong tendency of the radical to interact with other electrons turning the recipient in to a free radical. Cellular macromolecules such as lipids, proteins and nucleic acids are vulnerable to free radical damage.

Cheeseman KH and Slater TF (1993)[14] state that free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules and which are generally very reactive. They are produced continuously in cells either as accidental by -products of metabolism or deliberately during phagocytosis. The most important reactants in free radical biochemistry in aerobic

cells are oxygen and its radical derivatives (superoxide and hydroxyl radical), hydrogen peroxide and transition metals.

Young I.S (2001)[84] Free radicals are any molecular species capable of independent existence that contain an unpaired electron in an atomic orbital. Radicals are weakly attracted to magnetic field and are said to be para magnetic. These radicals are highly reactive and can either donate or extract an electron from other molecules, therefore behaving as oxidants or reductants. As a result of this high reactivity, free radicals have a very short half life (10-6 sec or less).

Claudia Dornelles Schneider and Alvaro Reischak de Oliveira (2004)[15] consider that oxygen free radicals are formed through the incomplete reduction of oxygen, generating species presenting high reactivity to other bio-molecules especially lipids and proteins of the cell membranes and even DNA. The injuries caused by oxidative stress present accumulative effects being related

to several diseases such as cancer, arteriosclerosis and diabetes.

Bin Zhao, Su-Yin Tham, Jia Lu et al (2004)[8] state that free radicals are highly reactive molecules that react with and damage cells through out the body. They are suspected of causing cardiovascular disease, cancer, neurological disorders, cataracts, arthritis, aging and other conditions such as muscle damage and fatigue.

MECHANISM OF PRODUCTION OF FREE RADICAL:

Peter H. Proctor and Edward S. Reynolds (1984)[57] Free radical are produced through the action of mono-oxygenase by various oxidative enzymes and by auto-oxidation mediated by heavy metals or quinones. This free radical when produced in unconfined or inappropriate manner plays a significant role in human disease.

Peter Wardman (1993)[56] Free radicals are important intermediates in natural processes involved in cytotoxicity, control of vascular tone and neurotransmission. Radiolysis

is a powerful method to generate specific free radicals. Oxygen is a common reactant in free radical processes, having a propensity to take part in single electron transfer or free radical reactions in which electrons become paired. The author has also stated that intra cellular levels of free heavy metals, particularly copper and iron, are critical in defining the extent of hydroxyl radical production from superoxide and hydrogen peroxide.

Loeckie L. DE Zwart, John H. N. Meerman, Jan N. M. Commandeur et al (1998)[46] Free radicals are continuously produced in all cells as by- products of metabolism, or for example, by leakage from mitochondrial respiration. The most important reaction of free radicals in aerobic cells involves molecular oxygen, and its radical derivatives (superoxide and hydroxyl radicals), peroxides and transition metals.

Pat Kendall (2000)[54] considers free radical production as a normal part of life, part of the equation of simply breathing in oxygen.

Young I.S (2000)[84] Free radical production occurs continuously in all cells as a part of normal cellular function. Free radical production can involve both endogenous and environmental factors. The various free radicals produced are superoxide, hydrogen peroxide, and hydroxyl radical. The presence of transition metals iron and copper accelerate the production of free radicals. Superoxide is produced by the addition of a single electron to oxygen. Any biological system generating superoxide will produce hydrogen peroxide as a result of dismutation reaction. Hydroxyl radical formation is by the decomposition of superoxide and hydrogen peroxide, catalyzed by transition metals.

Vasudevan DM and Sreekumari S. (2001)[79] Free radical may be formed by cleavage of covalent bond of a normal molecule, loss of singlet electron from a normal molecule, or addition of a singlet electron to a normal molecule.

FREE RADICALS IN DISEASE:

Sigmund A. Weitzman, Patrick W. Turk, Deborah Howard et al (1994)[67] stated that methylation of cytosine in DNA is important for the regulation of expression of many genes. During carcinogenesis, normal patterns of gene methylation can be altered by oxygen radical injury. These free radicals induce DNA strand breaks resulting in hypomethylation which has been associated with the development of cancer.

Barry Halliwell and Helen Wiseman (1996)[5] stated that reactive oxygen species (ROS) can produce gross chromosomal alterations in addition to point mutations and thus could be involved in the inactivation or loss of the second wild type allele of a mutated proto oncogenes or tumor suppressor gene that can occur during tumor promotion and progression allowing expression of the mutated phenotype.

The endogeneous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation,

depurination and deamination. Methylation of cytokines in DNA is important for the regulation of gene expression and normal methylation patterns can be altered during carcinogenesis. Conversion of guanine to 8- hydroxyl guanine, a frequent result of ROS attack has been found to alter the enzyme catalysed methylation of adjacent cytosines, thus providing a link between oxidative DNA damage and altered Methylation patterns.

Emmanuel C. Opara (1997)[22] in his study stated that smoking generates oxygen free radicals which play a causative role in the pathogenesis of chronic degenerative diseases like emphysema, lung cancer and coronary artery disease. Furthermore smoking causes lots of stress load, and that antioxidant supplements are helpful in preventing or mitigating oxidative damage.

John Cramer (1997)[41] stated that oxidants or oxygen containing molecules play an important role in causing cancer and antioxidant or free radical scavengers help to suppress cancer. It is also stated that cancerous cells

themselves may cause an overproduction of free radicals, acting as messenger molecules. Free radicals send signal through protein pathways which promote further uncontrolled cell growth. At molecular level it is stated that antioxidants may block signaling pathway which further blocks the process.

Wald N.J., Thompson S.G., Densem J.W et al (1998)[81] stated that singlet oxygen is toxic and is cancer inducing; this form of oxygen occurs as a result of many metabolic reactions.

Klaunig JE, Xug (1998)[43]-Oxidative stress results when the balance between productions of reactive oxygen species (ROS) overrides the antioxidant capability of target cell. ROS may interact with and modify cellular protein, lipid and DNA, which results in altered target cell function. Sustained chronic oxidative injury may lead to a non-lethal modification of normal cellular growth control mechanisms. Cellular oxidative stress can modify intercellular communication, protein kinase activity,

membrane structure, function, gene expression and result in modulation of cell growth specifically during promotion stage.

Loeckie L. DE Zwart, John H. N. Meerman, Jan N. M. Commandeur et al (1999)[46] Reactive free radicals formed within the cells can oxidize biomolecules and this may lead to cell death and tissue injury. Free Radicals (FR) cause oxidative damage to nuclear DNA and consequently somatic mutations such as base changes, deletions and chromosomal strand breaks are developed. Oxygen derived free radicals are important mediators of cell injury and cell death. These chemical species are directly or indirectly involved in various clinical disorders such as atherosclerosis, reperfusion injury, cancer etc.

Susan A. Keys and William F. Zimmerman(1999)[71] stated that photoreceptor cell membranes are vulnerable to oxidation .The potential for oxygen induced cellular damage may be greater in the retina because the transparency of the ocular structures allows light induced

generation of superoxide radicals. Membrane lipid peroxidation is one of the most prominent forms of cellular damage induced by conditions of oxidative stress, and the aerobic cells have evolved an arsenal of defenses against the injurious effects, the major being antioxidants.

Greg Kelly (2002)[28] stated the effects of cigarette smoking on lipid peroxidation and antioxidative enzymes like superoxide dismutases, glutathione peroxidase and catalase. Cigarette smoking induces lipid peroxidation and the antioxidant enzyme levels were enhanced in order to protect the tissues against the deleterious effects of the oxygen derived free radicals. The depletion of reduced glutathione in these organs could be due to its utilization by the tissues to mop off the free radicals.

Thomas PA, Devasagayam and Jayashree P Kamat (2002)[77]**-**Recent studies using newly developed detection methods show that singlet oxygen being generated in many biological systems can significantly and quite often adversely alter several crucial biomolecules including

DNA, proteins and lipids with undesirable consequences including cytotoxicity and or disease development.

Marion Dietrich, Gladys Block, Edward P. Norkus et al (2003)[47] Free radicals in cigarette smoke may cause oxidative damage to macromolecules such as lipids, proteins, and DNA contributing to the pathogenesis of cancer and cardiovascular disease. Decreased plasma antioxidant concentrations may indicate cigarette smoke related oxidative stress.

Syed Sultan Beevi, Muzib A. Hassanal Rasheed, Geetha (2004)[72] Free radicals play a key role in development of cancer by causing DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of proto oncogenes. Tobacco consumption in any form has demonstrated increase in free radical production leading to carcinogenic, teratogenic and genotoxic effects.

Linus Pauling (2005)[45] has stated that Oxidative damage accumulates in human tissues with age and can cause

degenerative diseases such as heart disease and cancer. Free radicals like hydroxyl radical cause damage to cell membranes by lipid per oxidation and the hydrogen peroxide may directly damage proteins and enzymes containing reactive thiol groups. Damage to DNA is implicated in cancer and oxidative damage to lipids plays an important role in atherosclerosis.

MECHANISM OF COUNTERACTING FREE RADICALS: Susan A. Keys and William F. Zimmerman (1999)[71] stated that membrane lipid per oxidation is one of the most prominent forms of cellular damage induced by conditions of oxidative stress, and the aerobic cells have evolved an arsenal of defenses.

Many water soluble antioxidants have been found to protect liposome's and biological membranes from lipid per oxidation. More commonly found antioxidants are ascorbic acid, cysteine, taurine and glutathione. By breaking the chain reaction of lipid per oxidation, these

antioxidants protect a much larger concentration of membrane lipids.

Chaudiere J. and Ferrari-Iliou R. (1999)[13] stated that intracellular antioxidants include low molecular weight scavengers of oxidizing species and enzymes which degrade super oxide and hydro peroxides. Such antioxidants prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biologic structures, added to this the antioxidants also play a major role in regulation of cellular metabolism and signalling.

Clifford D. Abiaka, Farida M. Al-Awadi, Hilal Al-Sayer et al (2001)[16] stated that micronutrient antioxidants except retinol decreased significantly in levels in smokers than non smokers, suggesting susceptibility to cigarette smoke oxidative stress. They concluded that micronutrient antioxidant depletions and altered associations may imply tumor utilization or antioxidant burden in oxidative stress or both.

Aghvani. T, Djalali, Keshavarz. A et al (2006)[1] stated that the extent of ROS induced damage can be exacerbated by decreased efficiency of antioxidants defense mechanisms. Therefore, it is important to pay attention to antioxidant defense mechanisms. Vitamin-C is a chain breaking antioxidant that reacts directly with super oxide singlet oxygen. Vitamin-E is a lipid soluble antioxidant present in plasma and erythrocyte membrane that prevent cellular damages by inhibiting DNA breakage induced by the reactive oxygen metabolites

Pat Kendall (2000)[54] stated that Free radical production is actually a normal part of life, part of the equation of simply breathing in oxygen. Usually, the body's natural defense systems neutralize free radicals that develop, rendering them harmless. Environmental assaults on the body can overpower the body's ability to neutralize free radicals rendering them to cause damage to structure and function of body's cells. Hence from warding off heart diseases to slowing degeneration of the brain and the eyes,

health benefits of antioxidants are indispensable. Antioxidants work by neutralizing highly reactive, destructive compounds called free radicals.

VARIOUS ANTIOXIDANTS:

David J. Hunter, Jo Ann E. Manson, Graham A. Colditz, et al; (1980)[20] stated that vitamin E, vitamin C, and vitamin A, have antioxidant properties, decrease the risk of cancer in general, but vitamin A, a regulator of cell differentiation have been hypothesized to reduce the risk of breast cancer.

Shklar G, Schwartz J, Trickler D et al (1992)[66] studied the effectiveness of a mixture of beta carotene, alpha tocopherols, glutathione and ascorbic acid for cancer prevention and stated that a mixture of these antioxidants produced a significant synergistic chemoprevention for oral cancer.

Gary Null (1994)[25] stated that as a water soluble antioxidant, vitamin C is in a unique position to

"Scavenge" aqueous peroxyl radicals before these destructive substance have a chance to damage the lipids .It works along with vitamin-E, a fat soluble antioxidant, and the enzyme glutathione peroxidase to stop free radical chain reactions.

Russel J. Reiter (1995)[61] The potential cause of age related destruction of neuronal tissue is toxic free radicals that are a natural result of aerobic metabolism. The brain is more susceptible to damage because it generates more free radicals per gram of tissue than any other tissue in the body. The major defense the brain uses is via enzymatic metabolism. Another way is via antioxidants, vitamin E and vitamin C, which protects the brain from oxidative stress by directly scavenging the toxic radicals.

James M. May(1999)[39] stated that ascorbic acid or vitamin-C, is a primary antioxidant of the plasma and within the cell, it can also interact with the plasma membrane by donating electrons to alpha tocopheroxyl

radical and a trans plasma membrane oxidoreductase activity.

Chow CK (2004)[12] stated the ability of dietary vitamin-E to mediate mitochondrial super oxide generation. Thus by decreasing the levels of reactive oxygen species, nitrogen species, vitamin E not only protects oxidative damage, but also modulates the expression of redox sensitive biological response modifiers that regulate important cellular events.

FOOD SOURCES OF ANTIOXIDANTS:

James W. Anderson, Tammy J. Hanna, Xuejun Peng et al (2000)[40] has studied the relationship between the intake of whole grain foods and the risk of heart disease. They have found that the foods that are rich in dietary fiber including fruits, vegetables, legumes and whole grain cereals tend to be a rich source of vitamins, minerals, phytochemicals, micronutrients and antioxidants. They have found that people who consume lot of whole grain cereals rich in antioxidants are at lower risk for heart diseases.

Bente L. Halvorsen, Kari holte, Mari C. W. Mybrstad et al (2002)[10] have assessed systematically the total antioxidants in a variety of dietary plants including various fruits, berries, vegetables, cereals, nuts and pulses. They found that plants that contain most antioxidants belong to Rosaceae family (dog rose, sour cherry, black berry, straw berry, raspberry), Empetraceae (crow berry), Ericaceae (blue berry), Grossulariaceae (black currant), Juglandaceae (walnut), Asteraceae (sunflower seed), Punicaceae (pomegranate) and Zingiberaceae (ginger). Fruits, berries and cereals contributed 43.6%, 27.1% and 11.7% respectively. Vegetables contributed only 8.9%.

Steinar Dragland, Harukisenoo, Kenjiro Wake et al (2003)[70] has assessed the contribution of culinary and medicinal herbs to the total intake of dietary antioxidants. They assessed that most berries, walnuts, sunflower seeds, ginger and pomegranates are among the high antioxidant dietary plants. They have found that dried culinary herbs tested, oregano, sage, peppermint, lemon balm, clove, all

spice and cinnamon contained very high concentrations of antioxidants(>75mmol/100gm)and be a better source of dietary antioxidants than many other food groups.

Arne Svilaas, Amrit Kaur Sakhi, Lene Frost Andersen et al (2004)[2] have determined the contribution of various food groups to total antioxidant intake and also assessed the correlations of total antioxidant intake from various food groups with plasma antioxidants. Intake of coffee contributed 11.1mmol followed by fruits (1.8mmol), tea (1.4mmol), wine (0.8mmol), cereals (0.8mmol) and vegetables (0.4mmol).The frequency of coffee intake had a major contribution to the total antioxidant intake.

Nancy Nairi Maserejian, Edward Giovannucci, Bernard Rosner et al (2006)[51] have prospectively evaluated fruit and vegetable consumption and the incidence of oral premalignant lesion. They have found that the risk of oral premalignant lesions was significantly reduced with higher consumption of fruits, particularly citrus fruits and juices,

while no consistent associations were apparent for vegetables.

ROLE OF VITAMIN-E AND VITAMIN-C IN ORAL DISEASES:

Shklar G, Schwartz J, Trickler DP et al (1987)[73] have found that Vitamin-E was shown to regress established epidermoid carcinomas of Syrian Hamster buccal pouch following tumour induction by application of 0.5% 7,12-dimethyl benz anthracene in mineral oil for 13 weeks. Vitamin-E was injected in to the tumour bearing buccal pouch twice weekly for 4 weeks in a dose of 250 micrograms. Microscopic examination showed small epidermoid carcinomas with degeneration of tumour cells. The hamster buccal pouch cancer model presents similarities to human oral cancer including of same oncogene. These results offer hope for the chemotherapy of human oral cancer with the use of a relatively non-toxic agent injected locally.

Shklar G, Joel L.Schwartz, Diane P.Trickler et al(1990)[74] have conducted a study with Syrian Hamsters buccal pouch initiated for cancer with Dimethylbenz anthracene (DMBA), supplemented with Vitamin-E. It revealed a dense infiltrate of mono-nuclear cells adjacent to tumour sites with a large number of cytotoxic T lymphocytes and macrophages showing that Vitamin-E appears to prevent tumour formation by stimulating a potent immune response to selectively destroy tumour cells as they begin to develop in to recognizable microscopic foci of carcinoma.

Vishwa N singh and Suzanne K Gaby (1991)[80] have suggested that consumption of carotene rich vegetables and Vitamin-C rich fruits markedly reduced the risk of oral cancer. It has been found that the conversion of nitrites and nitrates in to carcinogenic nitrosamines and nitrosamides can be blocked by Vitamin-C and Vitamin-E invitro and invivo suggesting their role in reducing the risk of cancer. They hypothesized that the potential chemo preventive

action of Vitamin-C and Vitamin-E is their antioxidant action.

Gloria Grindley, Joseph K. McLaughlin, Gladys Block et al (1992)[27] have done an epidemiological study to show a reduced oral cancer risk with vitamin E use. Their study found a significantly reduced risk of oral and pharyngeal cancer with use of individual's supplements, particularly vitamin E supplements, regardless of sex, race, tobacco and alcohol use, education, or several markers of dietary status. One of the limitations of the study is imprecise information on the amounts of various vitamins consumed by the cases and controls.

Shklar G, Schwartz J, Trickler D et al (1993)[66] conducted a study to find the effectiveness of a mixture of beta carotene, alpha tocopherol, glutathione and ascorbic acid for cancer prevention. Beta-Carotene and glutathione provided greater levels of chemoprevention than Vitamin E as single agents. In contrast, vitamin C treatment produced no antitumor effect but increased tumor burden. This

mixture of antioxidants produced a significant synergistic chemoprevention of oral cancer.

Ramaswamy, Rao VR, Kumaraswamy SV and Anantha N (1996)[60] studied the Serum levels of vitamins A, C, E using spectrophotometer and vitamin B12 and folate using radio assay in oral Leukoplakia patients. They found that except for vitamin E, all other Serum vitamin levels were decreased. It has been proposed that Vitamin-C is required in collagen synthesis for protective encapsulation of tumors and that it may act to inhibit lysosomal glycosidases which are responsible for invasion.

Chan SWY and Reade PC (1998)[17] in their review article explained the mechanism Vitamin C in Oral Carcinogenesis. Vitamin-C maintains the components of the electron transport system, such as cytochrome P-450 and influences the microsomal hydroxylation and demethylation systems responsible for carcinogen detoxification.

Vitamin-C inhibits carcinogens by blocking the conversion of precursors or pro-carcinogens in to carcinogenic metabolites or ultimate carcinogens. Vit-C has the ability to block the formation of carcinogenic N-nitroso compounds from nitrites in food and tobacco. Oral bacteria are known to convert ingested nitrate to nitrite, allowing for further nitrosation in to N-nitrosamine and N-nitrosamides and Vit-c is necessary for prevention of nitrosation. Thus the inhibitory effect of Vit-C on chemically induced oral carcinogenesis is likely to be due to its ability to block the formation of N-nitroso compounds.

The systemic effect of absorbed Vit-C may act protectively against chemically induced oral carcinogenesis by maintaining liver detoxification of carcinogens, by delaying tumour induction because of its ability to inhibit malignant transformation and limit tumour size due to its pro-oxidant cytotoxic effects and by inhibiting prostaglandin synthesis.

Susan Taylor Mayne and Douglas E.Morse (2001)[75] have documented the efficacy of supplemental nutrients (Beta-Carotene, Vitamins-A, C, E and selenium) in the regression of oral pre-cancerous lesions.

Harinder and Garewal S (2004)[32] have stated that intake and supplemental use of Vitamin-E and beta carotene have been associated with a lowered risk of cancer. Smokers, whose habit is a major factor, have low beta carotene levels in oral mucosal cells when compared with non-Smokers. In several laboratory and animal model systems, these agents strongly inhibit oral carcinogenesis. Beta carotene and Vitamin-E produce regression of oral leukoplakia, a pre-malignant lesion for oral cancer. This has been shown in seven clinical trials: five with beta carotene alone, one with Vitamin-E and one with a combination of both.

George E.Kaugars, William T.Riley, Richard B.Brandt et al (2006)[30] have found in their study that chewing tobacco was associated significantly with the presence of

an oral lesion. They found that these smokeless tobacco users with epithelial dysplasia were slightly older and had a lower intake of Vit-C.

METHODS OF ESTIMATING ANTIOXIDANTS:

Harris Rosenkrantz and Ade T. Milhorat (1950)[35] utilized "Baird associates Infrared recording spectrophotometer" for establishing the relative purity of tocopherols and related compounds indented for biologic assay. In this analysis, certain absorption bands in relation to structural configuration were attempted. In this method characteristic bands were described for differentiating the tocopherols from their chemical products.

Nair P.P and Magar M.G (1954)[50] has devised a new method for determination of vitamin-E in blood, using Beckman model DU quartz spectrophotometer. This procedure has the advantage of having greater specificity and ease of operation and the sensitivity has been considerably enhanced. In this method alkali was added to

serum for the reason that chromogenic reagent used will be inactive with esters of vitamin-E present if any.

Wilson P.W., Kodicek E and Booth V.H (1962)[83] has described a procedure for the complete separation of tocopherols using gas liquid chromatography. The versatility and high resolving power of gas liquid chromatography suggested itself as a useful technique the analysis of mixture of tocopherols. As little as 0.2µm of tocopherol could be assayed either in mixtures or in a natural source. The assay gave results that agreed with those obtained by paper chromatography.

Dhariwal KR, Washko PW and Levine M (1990)[21] has described a method for detection of De-hydro ascorbic acid (DHA) using high performance liquid chromatography with colorimetric electrochemical detection. Samples were first assayed for AA, then reduced with 2, 3-dimercapto-1-propanol to convert de-hydro ascorbic acid in the sample to ascorbic acid and subsequently re assayed for total ascorbic acid. The DHA content was the difference between the two

measurements. The assay is highly sensitive and reproducible with both standards and biological samples and was used for routine detection of less than or equal to 1pmol per sample injection of DHA.

Moeslinger T, Brunner M, Volf I et al (1995)[49] has presented a method for measuring ascorbic acid in methanol/ tri-chloro acetic acid extracts prepared from human plasma after enzymatic oxidation of ascorbic acid to de-hydro ascorbic by ascorbate oxidase. Samples were assayed spectro -photometrically monitoring the kinetics of the concentration dependent absorbance changes of de-hydro ascorbic with phosphate-citrate-methanol buffers. Ascorbic acid was determined as the difference between de-hydro ascorbic acid and total ascorbic acid content. Comparison of spectro-photometric determination with chromatographic procedure gave physiologically relevant concentrations.

Lee W, Roberts SM and Labbe RF (1997)[44] have developed a new procedure that will permit the automated

determination of plasma ascorbic acid determination with Roche Fara centrifugal analyzer. This procedure allows much faster throughput than conventional High Performance Liquid Chromatography (HPLC) methods while yielding results that correlate well and provide improved precision. This method has the disadvantage of being labor intensive and costly for a clinical laboratory.

Esteve MJ, Farre R, Frigola et al (1997)[23] has performed liquid chromatography method with U-V detection for measuring ascorbic acid (AA) and de-hydro ascorbic acid (DHA) in human blood and serum. This method used reversed phase column and cetyl trimethyl ammonium bromide as an ion pairing agent. AA was measured before and after the reduction of DHA with dithiothreitol. The analytical parameters, linearity, accuracy and precision show that the method is reliable and adequate for measuring the total vitamin-c content in serum and plasma.

Ihara H,Shino Y,Aoki Y et al(2000)[38] has developed a simple and rapid analysis of total ascorbic acid(AA) in

serum and plasma by automated enzymatic method. AA is oxidized by ascorbate oxidase to DHA that then reacts with o-phenylenediamine to form a quinoxaline derivative that absorbs at 340nm.The change in absorbance is directly proportional to the total AA concentration. On comparing, the manual enzymatic procedure gave lower values than those of the automated enzymatic procedure. This method correlated well with HPLC method. An experienced analyst can perform about 24 manual assays per hour whereas the automated procedure gave a rate of 100 assays per hour.

Sam A. Margolis, Mark Vangel and David L. Duewer (2003)[63] evolved a methodology to assist laboratories in improving the accuracy of their measurements and evaluating their quality control practices; National Institute for Standardization and Testing (NIST) has prepared and certified Standard Reference Material (SRM970, AA) in human serum. This SRM consists of two types of samples, level I, containing a low AA concentration and level II, containing a high AA concentration, that are at

approximately the 25th and 75th percentiles, respectively, of the distribution of AA concentrations in humans.

Bin Zhao, Su-Yin Tham, Jia Lu et al (2004)[8] has proposed High Performance Liquid Chromatography (HPLC) method with photodiode-array detection for simultaneous determination of vitamin-C, E and beta carotene in human plasma after oral administration of antioxidant supplement capsules to human subjects. This method uses isocratic chromatographic elution that separates and quantifies the water soluble vitamin-c and fat soluble vitamin-E and A.

MATERIALS AND METHODS

Selection criteria

Seventy five patients belonging to both sexes between the age group 50-70 years attending the out patient department of Meenakshi Ammal Dental College and Hospital, private clinics and govt. hospital Royapettah in Chennai were included in the study.

These patients comprised the following sub groups.

a) Group A: Apparently normal individuals with no history of tobacco related habits or clinical evidence of oral lesions.

b) Group B: Patients with history of tobacco related habits but without clinical evidence of oral lesions

c) Group C: Patients having tobacco related habits with oral pre cancerous lesions (Leukoplakia, oral sub mucous fibrosis)

d) Group D: Patients having tobacco related habits with established oral carcinoma.

Patients belonging to Group C and Group D represent histopathologically confirmed cases.

The Patients with diabetes, hypertension and other systemic diseases were excluded from this study.

The blood samples and clinical data were collected after informed consent.

Collection of Specimen

After the placement of tourniquet, the site of blood withdrawal was cleaned with cotton dipped in spirit. Venous blood was withdrawn from the anticubital vein, and it was transferred to a test tube which contained few drops of heparin. This sample was centrifuged at 3000 rpm for ten minutes, to obtain plasma.

Equipments used

a) Centrifuge : REMI RC4 Lab equipments

b) Cyclomixer : REMI CM 101 Cyclomixer

c) Spectrophotometer: Spectrophotometer 106

Apparatus required

a) Test tubes with standards

b) Pipettes

c) Water bath

d) Micro pipettes

e) Reagents

Estimation of Vitamin - E (Method of Desai):

Reagents for the estimation of Vitamin-E was prepared manually according to Desai's method (Annexure-I) and added to plasma. Spectrophotometer was used to estimate the Vitamin-E level at Optical Density (OD) of 536nm.

Estimation of Vitamin-C (Jacob's Method):

Reagents for the estimation of vitamin-C was prepared manually according to Jacob's method (Annexure II) and added to plasma. Spectrophotometer was used to estimate the Vitamin-C level at Optical Density (OD) of 520nm.

Statistical Analysis:

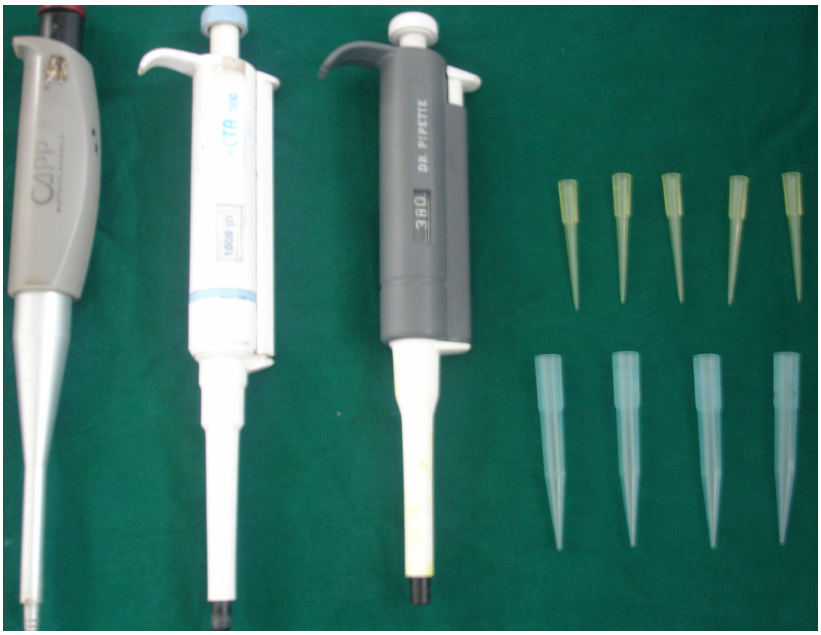
The data were analyzed by using one way analysis of variance (ANOVA), Turkey's multiple range test, mean and standard deviations.

ARMAMENTARIUM

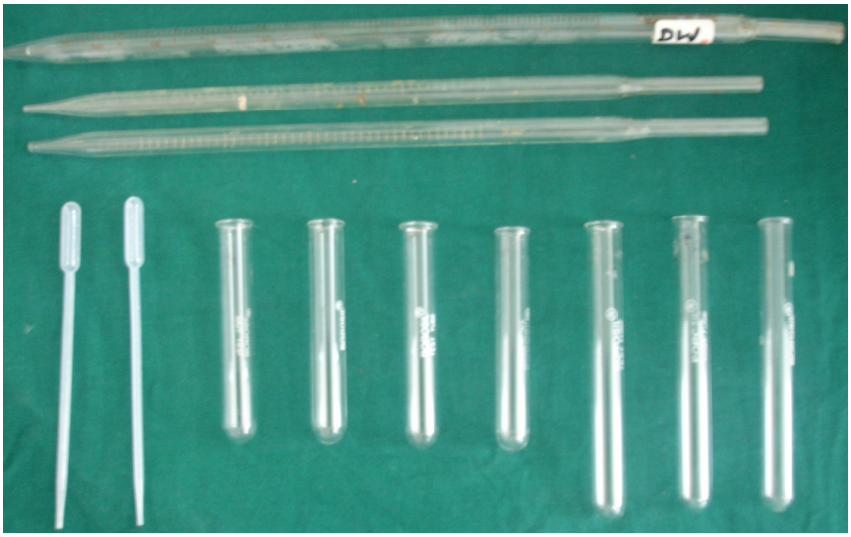
SAMPLE COLLECTION

CENTRIFUGE

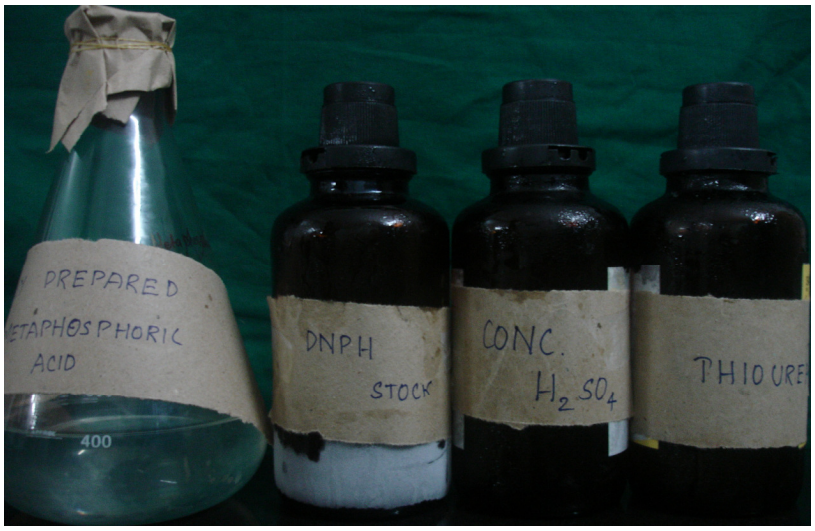
MICROPIPETTES



PIPETTES AND TEST TUBES



CHEMICALS USED FOR VITAMIN-E ESTIMATION



CHEMICALS USED FOR VITAMIN-C ESTIMATION

CYCLOMIXER AND HOT WATER BATH

SECTROPHOTOMETER

CLINICAL PHOTOGRAPHS

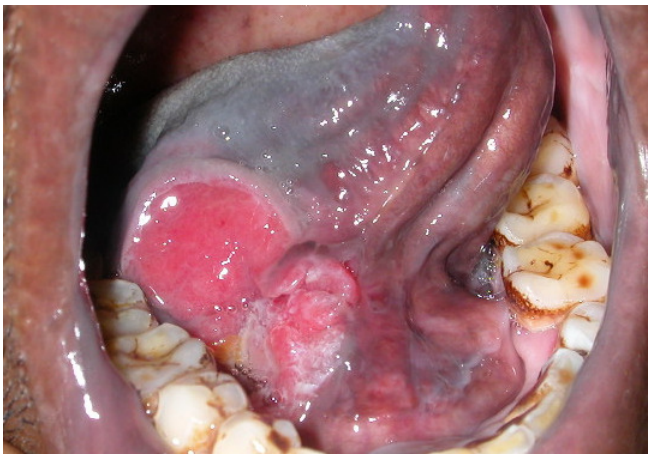
NORMAL MUCOSA

HABITS WITHOUT CLINICAL LESION

LEUKOPLAKIA

ORAL SUB-MUCOUS FIBROSIS

ORAL CANCER- TONGUE AND FLOOR OF THE MOUTH



RESULTS

A total of 75 cases comprised of the following groups were taken for this study.

e) Group A:

Apparently normal individuals with no history of tobacco habits or clinical evidence of oral lesions. It includes 15 cases, in which 6 were males and 9 were females.

b) Group B:

Patients with history of tobacco related habits but without clinical evidence of oral lesions. It includes 30 cases, in which 20 were males and 10 were females.

c) Group C:

Patients having tobacco related habits with oral pre cancerous lesions and conditions (Leukoplakia, oral sub mucous fibrosis) It includes 13 cases, in which 10 were males and 3 were females.

d) Group D:

Patients having tobacco related habits with established oral carcinoma. It includes 17 cases, in which 12 were males and 5 were females. (Table – 1).

Plasma Anti-Oxidant levels of Vitamin E and Vitamin C were estimated by Spectrophotometric analysis in these patients. The following were the results of Vitamin E level in controls and study groups:

1. In Group A, the vitamin E levels ranged from 0.1 to 1.4 and the mean was 0.8107mg/dl.

2. In Group B, the vitamin E levels ranged from 0.68 to 0.89 and mean was 0.8033mg/dl.

3. In Group C, the vitamin E levels ranged from 0.12 to 1.4 and mean was 0.7908mg/dl.

4. In Group D, the vitamin E levels ranged from 0.3 to 1.2 and mean was 0.7853mg/dl.(Fig-1).

The result shows that the values were not significant statistically between normal controls and different study groups as well as within the study groups (Table-2 and Fig-2).

The following were the results of Vitamin C level in controls and study groups:

1. In Group A, the vitamin C levels ranged from 0.4 to 0.7 and the mean was 0.4780mg/dl

2. In Group B, the vitamin C levels ranged from 0.29 to 0.41 and mean was 0.3573mg/dl

3. In Group C, the vitamin C levels ranged from 0.26 to 0.39 and mean was 0.3323mg/dl

4. In Group D, the vitamin C levels ranged from 0.01 to 0.36 and mean was 0.1313mg/dl (Fig3).

The result shows statistically significant values between normal controls and different study groups as well as within the study groups (Table-3 and Fig-4)

Comparison of Vitamin-E and Vitamin-C level shows statistically significant gradual reduction of Vitamin C level between patients with tobacco habits without lesions (group B), Individuals with tobacco related habits with precancer (group-C), individuals with tobacco related habits with oral cancer (group-D) compared to controls ; however, Vitamin E level does not show significant variation between these groups(Fig-5).

SEX DISTRIBUTION IN THE STUDY POPULATION

(TABLE-1)

	Controls	Habits with out clinical features	Habits with clinical features	Habits with oral cancer
Males	6	20	10	12
Females	9	10	3	5
Total	15	30	13	17

VITAMIN E LEVEL IN CONTROL AND STUDY GROUPS

TABLE-2

	N	Mean ± SD	F value*	Significant group**
Group A	15	0.8107±0.3660	0.035 P=0.996	Not significant
Group B	30	0.8033±0.05228		
Group C	13	0.7908±0.3464		
Group D	17	0.7853±0.2633		

Level of significance P>0.05

*** One way ANOVA ** Turkey HSD multiple range**

VITAMIN- C LEVEL IN CONTROL AND STUDY GROUPS

TABLE-3

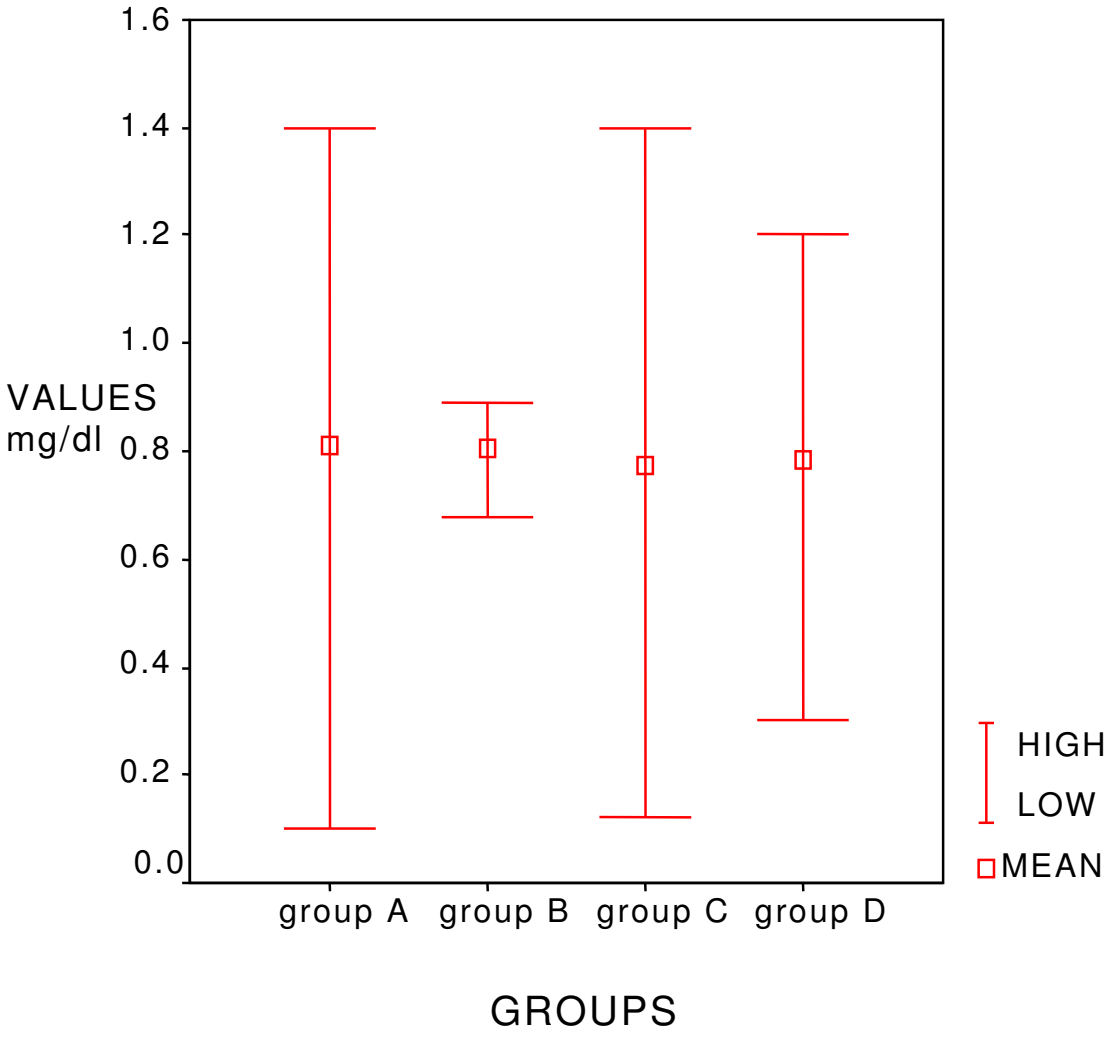
	N	**Mean ± SD**	**F value***	**Significant group****
Group A	15	0.4780 ±0.08231	85.860 P=0.000	A vs B A vs C A vs D B vs D C vs D
Group B	30	0.3573±0.03073		
Group C	13	0.3323±0.03563		
Group D	17	0.1313±0.09486		

Level of significance p < 0.05

*** One way ANOVA** Turkey HSD multiple range test**

VITAMIN- E LEVEL IN CONTROLS AND STUDY GROUPS

FIG: 1



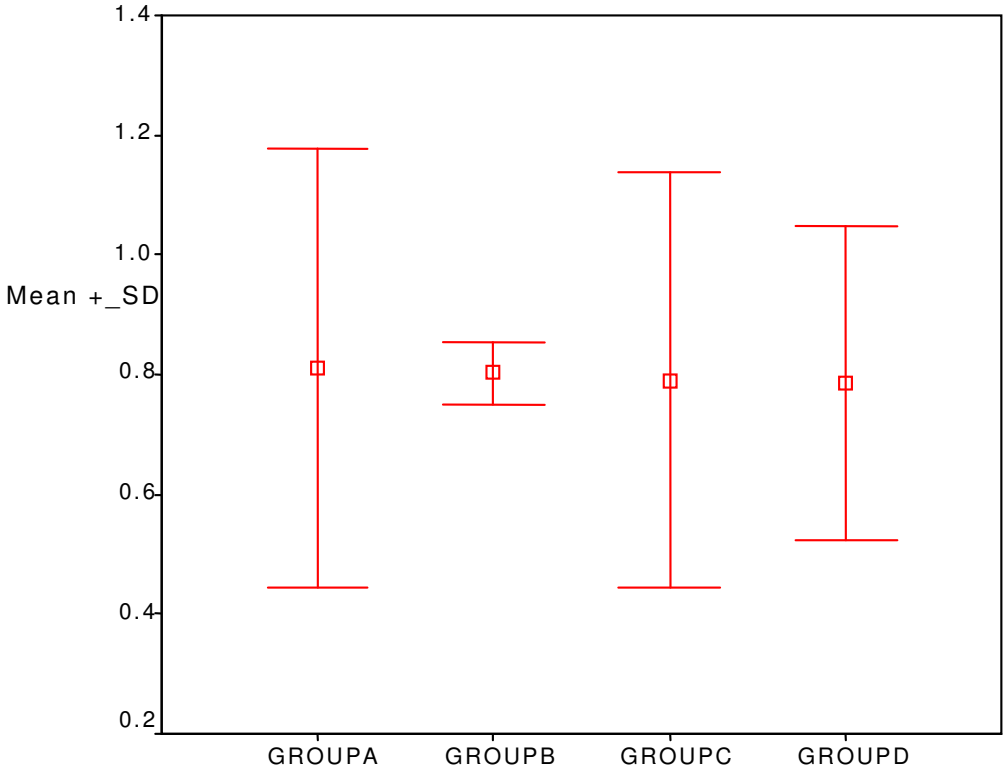
GROUP-A: Controls

GROUP-B: Habits with out clinical features

GROUP-C: Habits with precancer

GROUP-D: Habits with cancer

CONFIDENCE INTERVAL WITH MEAN FOR VITAMIN E LEVELS IN CONTROL AND STUDY GROUPS FIG: 2



GROUP-A: Controls

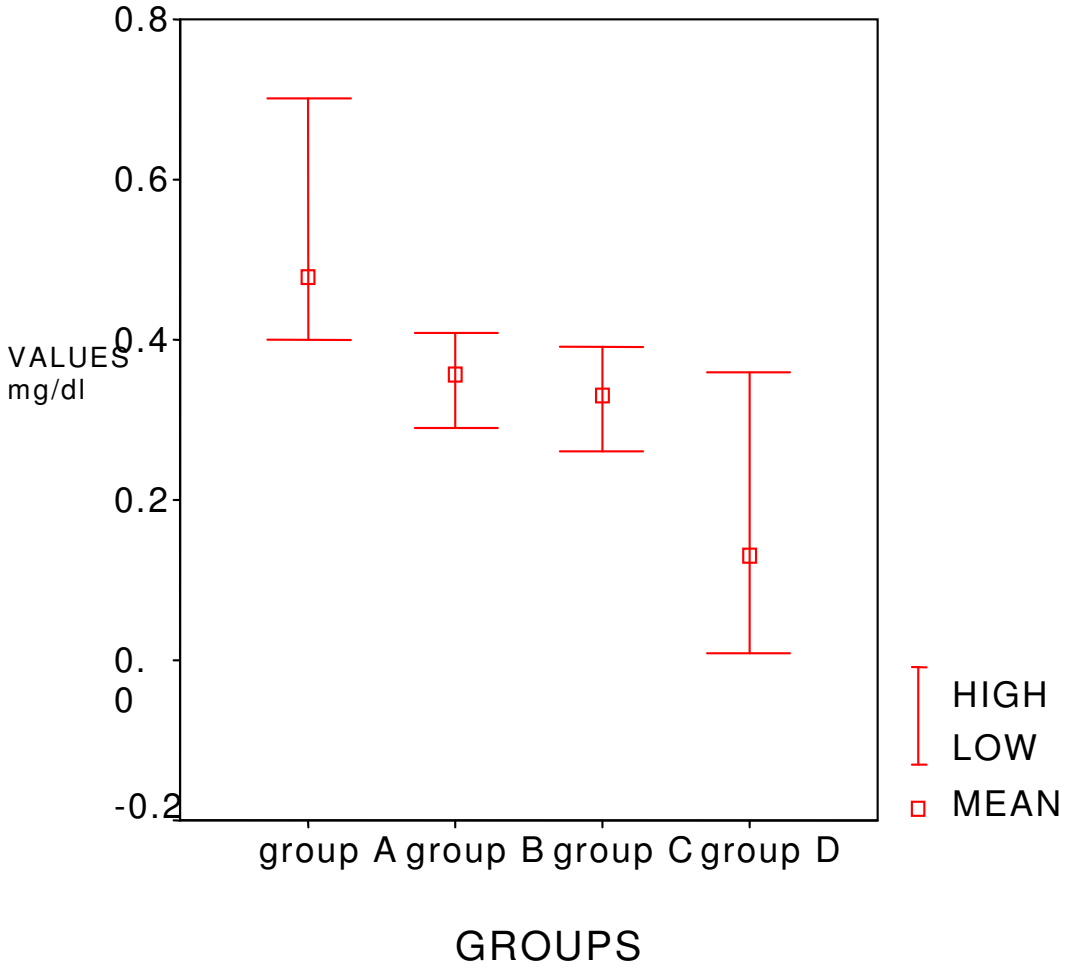
GROUP-B: Habits with out clinical features

GROUP-C: Habits with precancer

GROUP-D: Habits with cancer

VITAMIN C LEVELS IN CONTROLS AND STUDY GROUPS

FIG: 3



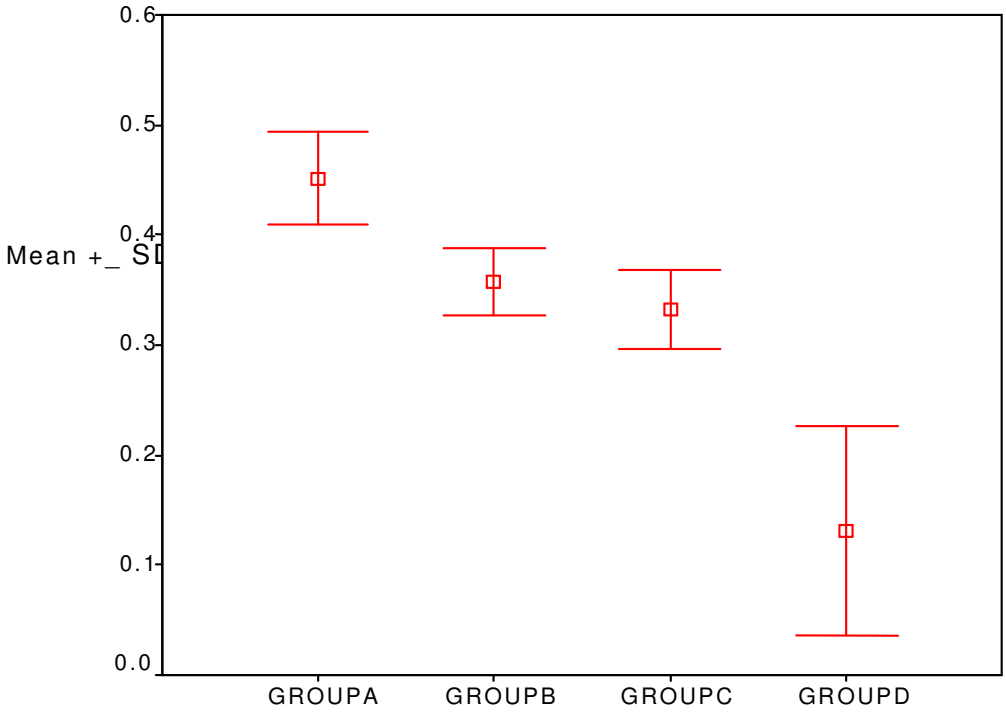
GROUP-A: Controls

GROUP-B: Habits with out clinical features

GROUP-C: Habits with precancer

GROUP-D: Habits with cancer

CONFIDENCE INTERVAL WITH MEAN FOR VITAMIN C LEVELS IN CONTROL AND STUDY GROUPS. FIG:4



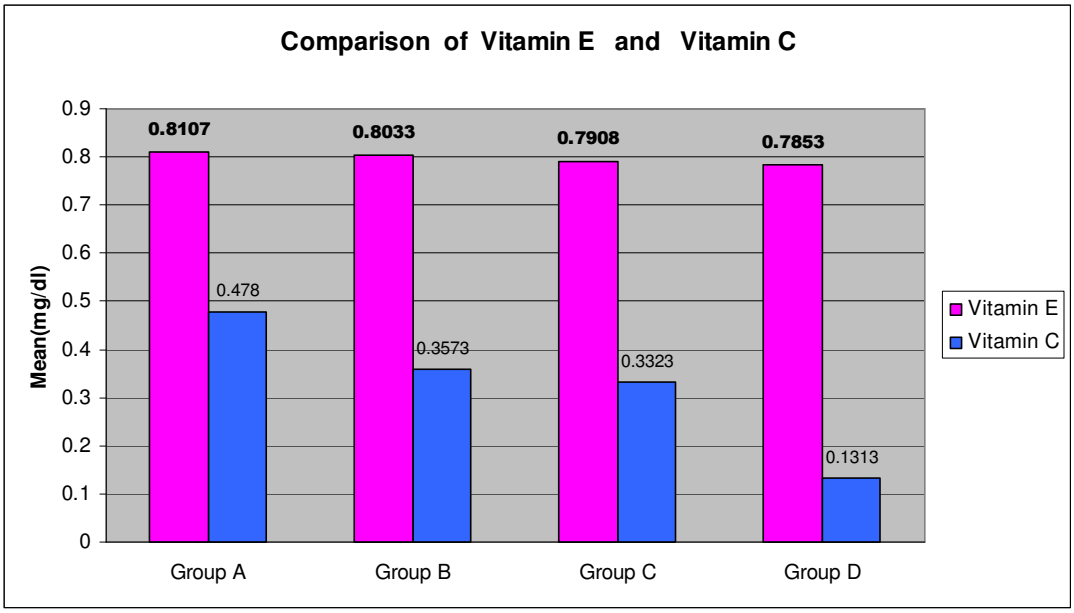
GROUP-A: Controls

GROUP-B: Habits with out clinical features

GROUP-C: Habits with precancer

GROUP-D: Habits with cancer

COMPARISON OF VITAMIN-E AND VITAMIN-C LEVELS IN STUDY AND CONTROL GROUPS:FIG:5.



DISCUSSION

Oxygen, an element indispensable for life can under certain situations have severely deleterious effect on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds known as Reactive Oxygen Species (ROS).These reactive species are called as free radicals **K.Bagachi and S.Puri (1998)[3].**

A free radical can be defined as any molecular species capable of independent existence that contain an unpaired electron in an atomic orbital. They are highly reactive and can either donate an electron to or extract an electron from other molecules, therefore behaving as oxidants or reductants. They have a very short half life (10^{-6} sec). These are highly unstable molecules **(Young IS, Woodside JV 2001)[84].**

Free radicals are derived either from normal metabolic processes in the human body or from external sources such

as exposure to x-rays, ozone, cigarette smoking, air pollutants and industrial chemicals.

Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions include those involved in respiratory chain, phagocytosis, in prostaglandin synthesis and in the cytochrome P450 system. Non- enzymatic reactions include reactions of oxygen with organic compounds as well as those initiated by ionizing radiations.

Bagchi K and Puri S (1998)[3] classified the sources of free radicals into two groups:-(I)internally generated sources of free radicals that include : Mitochondria, Phagocytes, Reactions involving iron and other transition metals, Arachidonate pathway, Peroxisomes, Exercise and Inflammation. (II) Externally generated sources of free radicals that include: Cigarette smoke, Environmental pollutants, Radiation, UV light, certain drugs, Pesticides,

Anaesthetics, Ozone. If free radicals are not inactivated, their chemical reactivity can damage all cellular macromolecules including proteins, carbohydrates, lipids and nucleic acids. Free radical damage to DNA is implicated in the causation of cancer.

In this study, we have chosen individuals with tobacco related habits, in which tobacco either in smoked or smokeless form is considered as externally generated source of free radical capable of inducing DNA damage. Individuals with these habits with clinically visible precancerous lesions and histologically confirmed Oral Squamous Cell Carcinoma were taken to study antioxidants levels in their plasma.

Young IS and Woodside JV (2001)[84] considered antioxidants as substances which when present in low concentrations compared to those of oxidizable substrate will significantly delay or inhibit oxidation of that substrate. The physiological role antioxidants is to prevent damage to cellular components arising as a consequence of

chemical reactions involving free radicals. Extensive ranges of antioxidant defenses both endogenous and exogenous are present. They can be divided in to three main groups. They are: 1. Antioxidant enzymes: They catalyse the breakdown of free radical species, usually in the intracellular environment. Example: a) Catalase-catalysis the two stage conversion of hydrogen peroxide to water and oxygen. b) Glutathione Peroxidase-catalyse the oxidation of glutathione at the expense of a hydroperoxide or another species such as lipid hydroperoxide. The predominant sub-cellular distribution of glutathione peroxidase in the cytosol and mitochondria suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in these locations. c) Superoxide Dismutase-catalyse the dismutation of superoxide to hydrogenperoxide. 2. Chain breaking antioxidants: Example: Vitamin-E, Vitamin-C. Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable by-products. The charge associated with the presence of an unpaired electron

becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing the further propagation of the chain reaction. 3. Transition metal binding proteins: Transition metal binding proteins (ferritin, transferring, lactoferrin and cerruloplasmin) act by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical.

In this study we have estimated the levels of Vitamin-E and Vitamin-C – Chain breaking antioxidants in plasma of individuals with tobacco habits with various mucosal changes.

A common form of damage is the formation of hydroxylated bases of DNA, which is considered important in chemical carcinogenesis. This adduct formation interferes with normal cell growth by causing genetic mutations and altering gene transcription.

Tobacco is one of substance that plays an important role in the development of oral mucosal changes resulting in

various precancer and oral cancer. Tobacco is used either in the smoked or smokeless form in which it was chewed with other ingredients like betel leaf, arecanut, and lime. In this study, we have included individuals with both form of tobacco habit.

Sigmund A. Weitzman et al (1994)[67] Methylation of cytosines in DNA is important for the regulation of expression of many genes. During carcinogenesis, normal patterns of gene Methylation can be altered.

Prabal Pal, Harman, Puneet Sharma and Nitin Verma (2004)[58] reviewed the antioxidant status in oral health and stated that mouth is considered as the mirror of the body and it takes continuous stress. Antioxidants can protect human population from oral pre cancer and cancer and increase life expectancy.

The present study is done to investigate the levels of non-enzymatic fat soluble antioxidant Vitamin-E and water soluble antioxidant Vitamin-C in apparently normal

individuals, patients with habits without any oral lesions, patients with habits having oral pre-cancerous lesions, patients with habits having histologically proven oral cancer.

In a study done by **Clifford D. Abiaka et al (2001)**[16] all micro nutrient antioxidants except retinol, decreased significantly in levels in smokers than non-smokers suggesting the susceptibility to cigarette smoke oxidative stress.

On the contrary, according to the study done by **Marion Dietrich et al (2003)**[47] plasma alpha tocopherol – Vitamin E concentrations were not significantly different between smokers, passive smokers and non-smokers. **Ramaswamy, Rao VR, Kumaraswamy et al (1996)**[60] studied the Serum levels of vitamins A, C, E using spectrophotometer and vitamin B12 and folate using radio assay in oral Leukoplakia patients. They found that except for vitamin E, all other Serum vitamin levels were decreased.

These findings are similar to our observations in which the level of Vitamin-E did not show statistically significant results between controls and individuals with tobacco related habits.

According to the study done by **Patel PS et al (2001)**[55] plasma beta-carotene and Vitamin-E levels were lower in patients with oral pre cancer and untreated cancer patients. Lower plasma beta-carotene and Vitamin-E were observed in tobacco consumers as compared to non-consumers.

In our study, there is only a marginal decrease in the level of Vitamin-E in precancer and cancer patients compared to the controls. The reason could be attributed to the indirect participation of Vitamin-C in regenerating the tocopheroxyl radical, which forms when tocopherol reacts with oxygen free radicals. **Chan SWY and Reade PC (1998)**[17]**. Bagachi K and Puri S (1998)**[3] also considers that Vitamin-C has sparing effect on Vitamin-E as it regenerates Vitamin-E from tocopheroxyl radical after it has neutralized free radicals.

Vitamin-C or ascorbic acid is a water soluble Vitamin. This vitamin is a free radical scavenger and interacts with free radicals in the water compartment of cells as well as in the fluids between cells. It is considered to be one of the most important antioxidants in extra cellular fluids. Vitamin-C suppresses the formation of carcinogens such as nitrosamines and quinines.

Vitamin-C in combination with Vitamin-E is shown to inhibit prostaglandin E2 synthesis, a process which has been shown to stimulate tumor growth, promotion and influence migration and metastasis.

According to another study done by Omer Pelletier in the year 1968 and 1970, the Vitamin-C levels in plasma were significantly reduced in smokers compared to non-smokers.

According to the study done by **Brook.M and Grimshan J.J (1968)**[11] the plasma Vitamin-C concentration in heavy

smokers and moderate smokers are significantly lower than that of non-smokers.

According to the study done by **Gordon Schectman, James C Byrd and Raymond Hoffmann (1991)**[31] the mean serum Vitamin-C concentration was reduced significantly in smokers compared to non-smokers.

The results of our study correlates well with the observations of the previous studies in which they compared the level of plasma Vitamin-C in smokers and non-smokers.

Ramaswamy, Rao VR, Kumaraswamy et al (1996)[60] had undertaken serum estimation of different anti-oxidants like vitamins A, C, E using spectrophotometer and vitamin B12 and folate using radio assay in oral Leukoplakia patients. They found that except for vitamin E, all other Serum vitamin levels were decreased.

Chan SWY and Reade PC (1998)[12] considered that the systemic effect of absorbed Vitamin-C may act protectively against chemically induced oral carcinogenesis by maintaining liver detoxification of carcinogens.**Nancy Nairi Maserejian, Edward Giovannucci, Bernard Rosner et al (2006)**[51] have prospectively evaluated fruit and vegetable consumption and the incidence of oral premalignant lesion. They have found that the risk of oral premalignant lesions was significantly reduced with higher consumption of fruits, particularly citrus fruits and juices.

In our present study Vitamin-C was found to be markedly reduced in patients with cancer and significantly reduced in patients with pre-cancer when compared to that of the controls.

Comparison of Vitamin-E and Vitamin-C in our study showed a statistically significant reduction in Vitamin-C in all the groups when compared to normal controls. On the contrary, Vitamin E level did not show significant variations in different study groups when compared to

normal controls. We concur with the opinion that Vitamin-C has sparing effect on Vitamin-E as it regenerates Vitamin-E from tocopheroxyl radical after it has neutralized free radicals.

SUMMARY AND CONCLUSION

1. This study comprised of 75 cases that included apparently normal individuals with no history of tobacco habits or clinical evidence of oral lesions, patients with history of tobacco related habits but without clinical evidence of oral lesions, patients having tobacco related habits with oral pre cancerous lesions and conditions (Leukoplakia, oral sub mucous fibrosis), patients having tobacco related habits with established oral carcinoma.
2. Plasma levels of Vitamin-E and Vitamin-C were estimated by Spectrophotometeric analysis in these patients.
3. We observed that the values of Vitamin-E were not significant statistically between normal controls and different study groups as well as within the study groups.
4. We observed that the values of Vitamin-C were statistically significant between normal controls and different study groups as well as within the study groups.

5. Comparison of Vitamin-E and Vitamin-C level shows statistically significant gradual reduction of Vitamin C level between patients with tobacco habits without lesions(group B),Individuals with tobacco related habits with precancer(group-C), individuals with tobacco related habits with oral cancer(group-D) compared to controls ; however, Vitamin E level does not show significant variation between these groups.
6. Comparison of our results with other research workers, we concur that Vitamin-C has sparing effect on Vitamin-E as it is considered to regenerate Vitamin-E from tocopheroxyl radical after it has neutralized free radicals, .

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ANNEXURE-I

Estimation of Vitamin-E (Method of Desai)

Reagents

Stock - α tocopherol 1mg/ml. 50mg was dissolved in 50ml of ethanol.

Standard:

25µl of stock was made up to 5ml with ethanol - 0.5mg

50 µl of stock was made up to 5ml with ethanol - 1 mg

100 µl of stock was made up to 5ml with ethanol - 2mg

200 µl of stock was made up to 5ml with ethanol - 4mg

$FeCl_3$ - 14mg of $FeCl_3$ was dissolved in 50ml ethanol

- 27.3mg of $FeCl_3$ was dissolved in 100ml ethanol

Ortho-H_3PO_4 - 50ml of H_3PO_4 was dissolved in 20ml of ethanol.

Bathophenanthroline - 20mg was dissolved in 10ml of ethanol.200 mg was dissolved in 100ml ethanol.

3ml of petroleum ether was added to 1ml of ethanol and 0.5ml of plasma sample .It was mixed well for 3 - 5 minutes in a cyclomixer. It was centrifuged for 10 min at 2500rpm. There were two layers which were separated after centrifugation. 2ml of the ether layer (top layer) was

pipetted into a fresh tube and was left at room temperature. It was left overnight for evaporation. Next day it was made up to 3ml with ethanol and was mixed well.

	Blank	**S_1**	**S_2**	**S_3**	**S_4**	**Test**
Standards	-	0.5ml	0.5ml	0.5ml	0.5 ml	-
Conc *mg%*	-	0.5	1	2	4	-
Ethanol	3ml	2.5ml	2.5ml	2.5ml	2.5ml	3ml
Batho	0.2 ml					
$FeCl_3$	0.2 ml					
	Mixed well for 1 minute					
H_3PO_4	0.2 ml					

The OD was read at 536nm.

ANNEXURE- II

ESTIMATION OF VITAMIN-C (JACOB'S METHOD)

Reagents

a. Metaphosphoric acid solution, (6.0g/dl) 30.0 gm of metaphosphoric acid (Hpo_3) was dissolved in distilled water and was brought to a final volume of 500 ml, and it was prepared just before use.
b. Sulphuric acid (4.5 mol/liter) 250 ml of concentrated sulphuric acid, of reagent grade, was added slowly to 500 ml of cold water in glass flask, it was then cooled and filled to mark with distilled water. Since significant heat was generated when concentrated sulphuric acid was diluted, the flask was placed in an ice bath. The concentrated acid was added slowly and the resulting solution was mixed constantly.
c. **Sulphuric acid (12 mol/litre)** 650 ml of concentrated sulphuric acid was added slowly to 300 ml cold water in a 1 litre flask.
d. **2,4 Dinitrophenylhydrazine reagent (2.0g/dl) in sulphuric acid, (4.5mol/litre)** 10g of 2,4 dinitrophenylhydrazine was dissolved in sulphuric acid 4.5 mol/ litre and was diluted to a final volume of 500ml, it was left to stand in the refrigerator overnight and was then filtered.

e. **Thiourea solution(5.0 g/dl)** 5g of thiourea was dissolved in distilled water and was diluted to a final volume of 100ml.
f. **Coppersulphate solution (0.6g/dl)** Anhydrous coppersulphate was dissolved in distilled water and was diluted to a final volume of 100ml.
g. **Dinitrophenylhydrazine - thiourea - coppersulphate reagent (DTC) reagent.** 5ml of thiourea solution, 5 ml of coppersulphate solution, and 100 ml of 2,4 dinitrophenylhydrazine reagent were mixed and was Stored in a bottle at 4^{o}C. This reagent was used for a maximum of 1 week.
h. **Calibrators** All ascorbic acid calibrators were prepared daily

a. Vitamin-C stock calibrators (50.0 mg/dl)

50mg of ascorbic acid was dissolved in metaphosphoric acid (6.0g/dl) and brought to a final volume of 100ml with metaphosphoric acid.

b.Intermediate vitamin-C calibrators (5.0 mg/dl)

10.0 ml of stock calibrator was pipetted into 100ml volumetric flask and was diluted to mark with metaphophoric acid (6.0 g/dl)

c. Working Calibrators

The following amounts of intermediate calibrator: 0.5, 2.0, 4.0, 6.0, 10.0,15.0 and 20.0 ml,were pippeted in to a

series of 25ml volumetric flasks, and was then brought to a final volume of 25ml with metaphosphoric acid (6.0 g/dl) to yield working calibrators of 0.10,0.40,0.80,1.20,2.00,3.00, and 4.00 mg/dl.

Procedure

1. 0.5 ml of heparinised plasma was added to 2.0 ml of freshly prepared metaphosphoric acid in a 13 x 10 mm test tube, and was mixed well in a vortex mixer; the plasma-metaphosphoric acid mixture was then centrifuged for 10 min at 2500 rpm. 1.2 ml of the clear supernatant was pippeted into a 13 x 100 mm Teflon lined, screw cap test tube.
2. 1.2 ml of each concentration of working calibrator was added in to 13 X 100 mm screw-cap test tubes. Calibrators were prepared in duplicate. 1.2 ml of metaphosphoric acid was added to two tubes for use as blanks
3. 0.4 ml of DTCs reagent was added to all tubes. The tubes were caped the contents were mixed, and the tubes were incubated in a water bath at 37°C for 3 hours.
4. The tubes were then removed from the water bath and were chilled for 10 min in an ice bath. 2.0 ml of cold sulfuric acid (12 mol/L) was added to all tubes and were capped, and mixed in a vortex mixer. (The temperature of the mixture did not exceed the room temperature.)

5. The Spectrophotometer was adjusted with the blank to read zero A at 520nm, and the calibrators were read. The concentration of each working calibrator was plotted versus absorbance values.

Calculation

The concentration of the sample obtained from the calibration curve was multiplied by 5 (this is to correct for the dilution of the plasma by metaphosphoric acid) to give the concentration of ascorbic acid per dl of plasma.

0.5 ml of Plasma (Heparin) + 2 ml of Meta Phosphoric acid

↓

(Mixed well in a vortex mixer)

Centrifuged at 2500rpm for 10 min

↓

1.2 ml of clear supernatant was pipetted into screw capped test tube

	B	S_1	S_2	S_3	S_4	S_5	S_6	T
Conc (mg %)	-	0.1	0.2	0.4	0.8	1.6	2	-
Standard (ml)	-	1.2	1.2	1.2	1.2	1.2	1.2	-
Metaphosphoric acid (ml)	1.2ml							
Test (ml)								1.2 ml
DTC s (ml)	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml	
the tubes were incubated in water bath at 37°C for *3* hour, The tubes were removed from water bath *&* chilled for 10 min in ice bath.								
Cold $H_2 SO_4$	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml

B: BLANK; S: STANDARDS; T: Test

Read at 520 nm using systronics spectrophotometer 106 (Digital)

$$\text{Concentration of sample} = \frac{\text{O.D of test}}{\text{O.D of Std}} \times \text{Concentration of Std}$$

OD: OPTICAL DENSITY